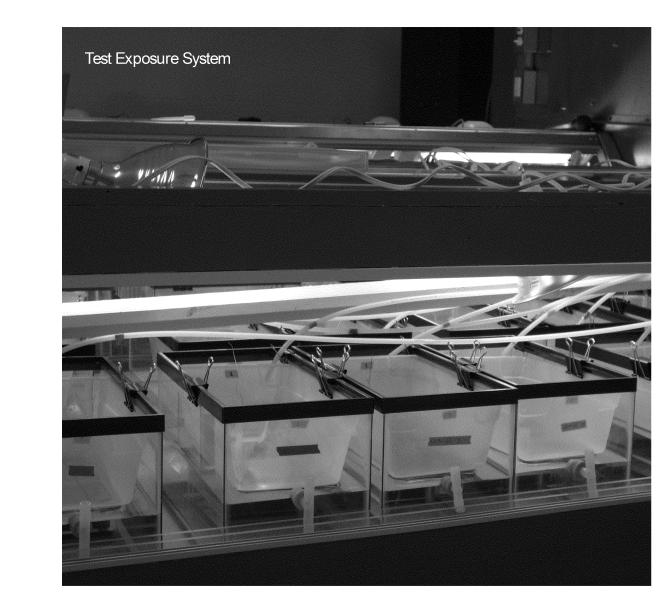
Sulfate Toxicity (Table 2)



ABSTRACT ENVIRON and Fort Environmental Laboratories performed a 21-day hydroponic experiment to determine the toxicity of

sulfate to wild rice seeds and seedlings. The effects of sulfate and chloride salts at equivalent osmotic potentials were evaluated to determine any adverse effects due to sulfate or osmotic stress. Sulfate-treated plants exhibited decreased root length, shoot length, leaf number and phytotoxic effects at sulfate concentrations of 5,000 mg/L. The time to 30 percent mesocotyl emergence (ET30) decreased at 2,500 mg/L sulfate, indicating a potential stimulatory effect. Sulfate exposures of less than 5,000 mg/L had no effect on five additional endpoints. Multiple regression analyses indicated that most observed changes could be attributed to osmotic effects rather than sulfate. Two exceptions were shoot length and leaf number, for which lower conductivity-based 25 percent inhibition concentration (IC25) values were observed for sulfate rather than chloride. Conversely, chloride was more toxic than sulfate as determined by root length and phytotoxicity. In summary, sulfate concentrations below 5,000 mg/L did not adversely affect early life-stage wild rice during a 21-day period, and effects at 5,000 mg/L sulfate were attributable to osmotic stress rather than sulfate toxicity in two of four endpoints.

INTRODUCTION

Wild rice (Zizania palustris L) is native to the Great Lakes region of North America. It has been a staple of the Native American diet for centuries, and provides food for waterfowl and wildlife. Although sulfate is a required nutrient for terrestrial and aquatic plants, field studies in Minnesota during the 1930s and 1940s suggested that surface water sulfate may influence the geographic distribution of wild rice at concentrations above 10 mg/L (Moyle 1994). In 1973, the Minnesota Pollution Control Agency adopted a statewide 10 mg/L sulfate water quality standard for the protection of wild rice. Understanding causal factors that negatively impact wild rice populations is desirable, yet few studies have examined the effects of sulfate on wild rice under controlled conditions. A hydroponic study was conducted to determine the impact of sulfate on seed germination and seedling development of wild rice under controlled laboratory conditions. It was hypothesized that sulfate does not adversely affect early life stages of wild rice until sulfate concentrations impose osmotic stress. Dose-response relationships of sulfate concentrations and wild rice germination, growth and development were used to determine the toxicity of sulfate in early life stages of Z. palustris. Seedlings were also subjected to osmotic conditions similar to those created by the sulfate treatments using chloride salts to determine whether sulfate was uniquely toxic to wild rice, or if observed effects

METHODS & MATERIALS Experimental Method

Water temperature 21 ± 2°C (light) and 12 ± 2°C (dark)

solutions. The pH was consistently between 6.1 and 7.2 standard unit (su).

were due primarily to osmotic stress.

Preliminary studies were conducted to determine a suitable test medium to hydroponically grow field-harvested wild rice from seed, identify reproducible and ecologically relevant test endpoints, and determine appropriate sulfate test concentrations for a good laboratory practices (GLP)-compliant study. A daily renewal hydroponic system utilizing a modified Hoagland's solution (HS-1; (Hoagland and Amon 1950); (Malvick and Percich 1993)) was selected to test the effects of sulfate and chloride salts on 10 endpoints in rice seeds and seedlings over 21 days. The modified HS-1 solution contained 50 mg/L sulfate, 25 percent ammonium (molar basis) in a mixture of ammonium and nitrate, and served as the base medium and diluent for all test exposures. A combination of sodium, potassium, calcium and magnesium salts of sulfate and chloride was used to prepare test solutions with monovalent to divalent salt ratios of 2:1. All salts were reagent-grade materials of greater than 98 percent purity. Additional key test conditions included: • Sulfate and chloride exposures prepared with a balanced cation and anion mixture to create equivalent osmotic potentials at each exposure level • 100 mg/L boron positive control to document plant response to a known toxicant • Nominal sulfate concentrations of 10,250, 1,000, 2,500 and 5,000 mg/L (0.8, 4.2,19.8, 51.1 and 103.2 meq/L) were evaluated for effects on wild rice • Five chloride exposures with the same sulfate concentration as the HS-1 control and the same osmotic strength as the four highest sulfate exposures (Table 1) • Exposure vessels: 10-L glass aquaria with temperature and photoperiod control Each of four test replicates consisted of two baskets, each containing 30 seeds One basket per replicate harvested at study day (SD) 10 and SD 21 • Static, daily renewal replacement of 70 percent test solution and control Incandescent and fluorescent plant growth lights - 16:8 hour light:dark photoperiod at 5,000 \pm 1,000 lux

• Dissolved oxygen and oxidation reduction potential readings confirmed that oxidizing conditions existed in test

Table 1. Physicochemical Characteristics of Sulfate and Chloride Treatments $(mg/L) \quad (mg/L) \quad (mg/L) \quad (\mu mhos/cm) \quad (mg/L) \quad (mg/L) \quad (mg/L) \quad (mg/L)$ (1.2) (15.5) (19.9) (1.0) (0.6) (0.2)
 4.2
 250
 10
 239 (3.7)
 1,729 (12.2)
 248 9 61.8 (4.8)
 61.8 (4.8)

 4.2
 (45.4)
 (1.5)
 (4.3)
 (0.1)
 1,044 2,797 589 8 57.0 4.6 (8.1) (18.0) (91.0) (1.8) (3.0) (0.1) 2,545 4,423 1,348 10 61.3 4.9 (12.6) (76.2) (10) (0.8) (1.3) (0.1) (19.1) (59.3) (352.9) (2.4) (1.8) (0.2)
 50
 1,960
 410
 9
 56.5
 4.8

 (1.2)
 (26.6)
 (75.2)
 (1.3)
 (1.2)
 (0.2)

 50
 2,906
 539
 8
 56.0
 4.9

 (1.7)
 (20.9)
 (94.4)
 (0.8)
 (3.0)
 (0.1)

 54
 4,224
 795
 7
 59.0
 4.8

 (1.1)
 (28.2)
 (173.6)
 (1.0)
 (0.4)
 (0.0)

^a Mean with standard error of the mean (SEM) in parentheses The following wild rice seed or seedling endpoints were assessed at SD 10 and SD 21. Seed activation: absorption of water by the seed to result in seed coat disruption Mesocotyl emergence: the appearance of plant tissue from germinated seed 30 percent mesocotyl emergence time (ET30) Seedling mortality: degradation of emerged plant tissue from germinated seeds • Shoot biomass: combined dry mass (105°C) of the mesocotyl, coleoptile, primary leaf and secondary leaves Root biomass: the combined dry mass of seminal roots and associated rootlets Root and shoot lengths were determined from digitized photographs Leaf number: the number of secondary leaves formed per seedling · Phytotoxicity, including leaf chlorosis, darkening of tissue, wilting and deformity Based on preliminary testing, minimum test acceptance criteria in the HS-1 control were: 95 percent seed activation

 53
 8,509
 1,468
 6
 56.0
 4.6

 (1.3)
 (59.8)
 (335.6)
 (0.5)
 (4.1)
 (0.1)

Statistical analyses (significance determined at alpha 0.05) utilized SigmaStat 11.2 and Minitab® 17.1.0. Initial analyses indicated strong similarities between SD 10 and SD 21 results, so SD 10 results were not reported. Seed activation and seedling survival were 100 percent in all treatments, so these endpoints were excluded from analysis. For endpoints exhibiting statistical significance in ANOVA, a Dunnett's post hoc separation of means test was applied. A Kruskal-Wallis median test was used to identify any significant differences across sulfate treatments for endpoints that produced nonparametric data. As a result of the good agreement between nominal and measured sulfate concentrations (+/-10 percent), nominal sulfate concentrations were used in NOEC and LOEC determinations. To determine toxicity based on osmotic effects, 25 percent inhibition concentration (IC25) values and 25 percent stimulatory concentration (SC25) values were determined using linear interpolation of conductivity data.

The boron positive control test acceptance criterion was greater than or equal to 80 percent phytotoxicity.

RESULTS

sulfate exposure was observed.

 30 percent mesocotyl emergence 90 percent seedling survival

Physicochemical Conditions (Table 1) • Average measured sulfate concentration in HS-1 control was 52+1.2 mg/L. • Average measured sulfate in test exposures was generally 90 to 110 percent of nominal. Average measured sulfate for chloride treatments was 50 to 54 mg/L. Acceptable agreement between average conductivity in chloride treatments and corresponding osmotic potential Control Performance The HS-1 negative control minimum performance criteria were met (95 percent seed activation, 30 percent mesocotyl emergence and 90 percent survival). The boron positive control 100 percent phytotoxicity rate indicated compliance with pre-determined test acceptability • Significantly reduced emergence, secondary leaf biomass and root length; an increased ET30 value further demonstrated the toxic effects of boron on developing wild rice. • Root biomass, shoot biomass, shoot length and leaf number did not differ significantly between boron-treated plants and the HS-1 control.

• Toxicity (LOEC) was observed at 5,000 mg/L sulfate for shoot length, root length, leaf number and phytotoxicity. • Stimulatory effects due to sulfate were observed for root length at 10 mg/L sulfate. The ET30 value was significantly reduced at 2,500 mg/L sulfate. • The endpoints unaffected by sulfate (NOEC = 5,000 mg/L) were seed activation, mesocotyl emergence and emergence time, seedling survival, shoot biomass and root biomass. Point Estimates (Table 2) Conductivity-based sulfate IC25 determinations were consistent with LOEC determinations. • Toxicity was observed only for shoot length, root length, leaf number and phytotoxicity (IC25 values ranged from 5,590 to 7,385 umho/cm).

• Root length and shoot length were the most sensitive endpoints. • Based on overlapping 95 percent fiducial limits, root length and shoot length IC25 values were not significantly different, and root length and shoot length IC25 values were significantly lower than those for leaf number and Based on non-overlapping 95 percent fiducial limits for IC25 values, sulfate was more toxic than chloride as determined by shoot length, whereas chloride was more toxic than sulfate as determined by root length and Sulfate SC25 value of 1,771 umho/cm for mesocotyl emergence corroborates the finding of sulfate stimulation of ET30 at 2,500 mg/L sulfate. Four of five chloride exposures had increased ET30 values relative to the HS-1 control indicating chloride delayed wild rice emergence. Root biomass and shoot biomass were stimulated by increased chloride concentrations (SC25 values 4,600 umho / cm and 7,054 umho/cm, respectively). • Sulfate SC25 values for these endpoints were greater than 8,507 umho/cm. Findings indicated that sulfate toxicity to wild rice occurred only at the highest sulfate exposure (5,000 mg/L), and • Three endpoints also exhibited chloride toxicity, indicating that osmotic effects are the cause toxicity rather than • Toxic effects on wild rice were likely sulfate-specific for shoot length, whereas chloride exhibited greater toxicity than sulfate based on root length and phytotoxicity.

occurred for root biomass and shoot biomass. Table 2. Summary of Concentration-Based Endpoints and Point Estimates

	Toxicity: Concentration-Based ^a Numerical Endpoints			Point Estimates ^b			
Measurement Endpoint	NOEC	LOEC		Sulfate		Chloride	
	(mg SO ₄ ²⁻ /L)	(mg SO ₄ ²⁻ /L)	p Value	Point	Estimate (umhos/cm)	Point	Estimate (umhos/cm
Seed Activation	5,000	>5,000	NDc	SC/IC25	>8,507 ^d	SC/IC25	>8,509 ^d
Mesocotyl Emergence ^e	5,000	>5,000	0.062	SC25	1,771 (1,431– 2,076)	SC/IC25	>8,509 ^d
30% Emergence Time ^f	5,000	>5,000	0.036	SC/IC25	ND	SC/IC25	ND
Seedling Survival	5,000	>5,000	ND °	SC/IC25	>8,507 ^d	SC/IC25	>8,509 d
Shoot Biomass	5,000	>5,000	0.073	SC/IC25	>8,507 ^d	SC25	7,054 (6,367– 7,741)
Shoot Length	2,500	5,000	0.004	IC25	5,780 (5,491– 6,069)	IC25	7,690 (7,306– 8,075)
Root Biomass	5,000	>5,000	0.301	SC/IC25	>8,507 ^d	SC25	4,600 (4,370– 4,830)
Root Length ⁹	2,500	5,000	<0.001	IC25	5,590 (5,311– 6,290)	IC25	1,870 (1,777– 1,964)
Leaf Number	2,500	5,000	<0.001	IC25	6,810 (6,470– 7,151)	SC/IC25	>8,509 ^d
Phytotoxicity e	2,500	5,000	<0.001	IC25	7,385 (7,271– 7,485)	IC25	6,476 (6,387– 6,555)

^e Emergence and phytotoxicity point estimates determined by probit analysis. Remaining point estimates determined by f 2,500 mg/L sulfate decreased the time to 30% emergence. Not determined (ND), data could not be fit to linear g Increased root length relative to HS-1 was found in 10 mg sulfate /L, Dunnett's test, p<0.001.

To further evaluate whether the effects of high sulfate concentrations on wild rice were specifically attributable to sulfate, or more generally to osmotic stress, regression analysis of conductivity and ion type was carried out for seven biological endpoints previously assessed for sulfate toxicity. For all seven endpoints, quadratic and / or linear regression models were overall statistically significant (Table 3). • Quadratic models accounted for a greater proportion of variation than linear models in five endpoints. • Variation in shoot length and leaf number were better described by linear models.

 Statistically significant components of each model differed by endpoint. • Ion type (chloride versus sulfate) was not significant overall, nor did the regression coefficients significantly differ by ion type, indicating no sulfate-specific effect on mesocotyl emergence or phytotoxicity (Figure 1 and 2). • Phytotoxicity occurred at salt concentrations imparting greater than 4,000 umho / cm conductivity, regardless of salt • Sulfate and chloride exposure affected root biomass differently, but both ions were detrimental to root length at similar ionic strengths (Figures 3 and 4). • Shoot biomass of chloride-treated plants increased in response to increasing conductivity, while sulfate-treated plant shoot biomass decreased at high conductivity (Figure 5).

Shoot length and leaf number (Figures 6 and 7) decreased similarly for both salt mixtures.

Multiple Regression: Chloride versus Sulfate Responses

Table 3. Multiple Regression of Conductivity and Ion Type on Measurement Endpoints ^a Parameter Emergence Root Biomass Root Length Biomass Shoot Length Leaf Number Phytotoxici

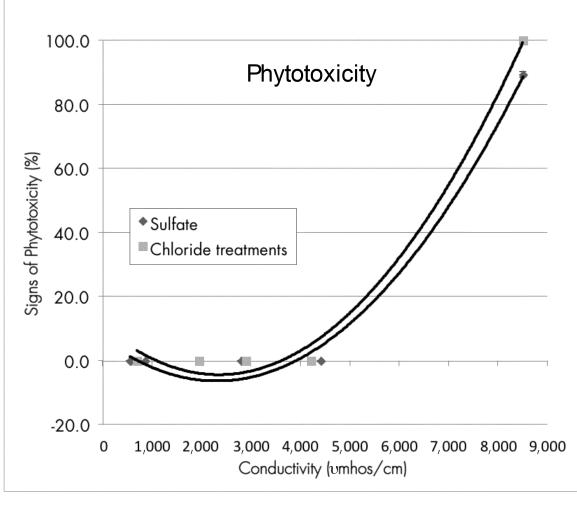
• Sulfate-specific stimulatory effects occurred for mesocotyl emergence, whereas chloride-specific stimulatory effects

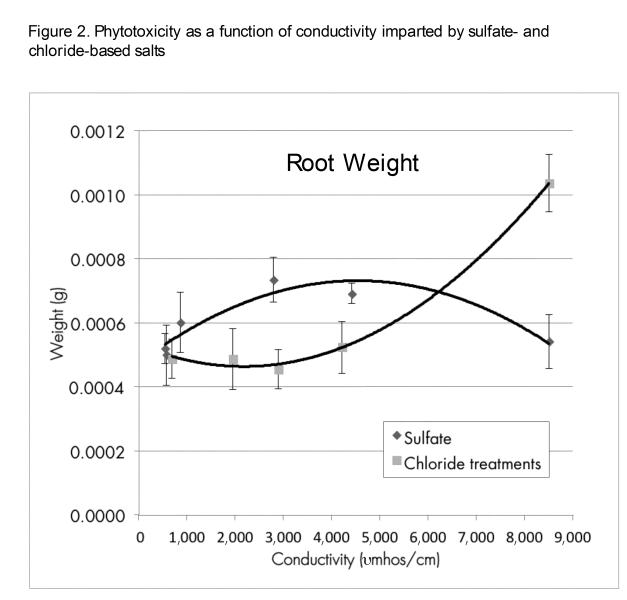
Sulfate Chloride 25.8 (20.6) 0.000426 (20.6) 4.72 (20.00763) 0.00763 (20.6) 3.55 (20.6) 3.89 (20.6) B 0.025 (20.6) 0.463 (20.6) 0.002 (20.6) 0.480 (20.6) 0.001 (20.6) 0.140 (20.6) Sulfate Chloride 0.0167 (20.6) 1.23 x 10 (20.6) 2.43 x 10 (20.6) 8.25 x 10 (20.6) -0.000172 (20.000154) 0.000154 (20.6) A 0.028 (20.6) 0.036 (20.6) 0.022 (20.6) 0.069 (20.6) -8.74 x 10 (20.6) -3.79 x 10 (20.6) A 0.028 (20.6) -1.28 x 10 (20.6) -3.32 x 10 (20.6) -9.01 x 10 (20.6) NA (20.6) NA (20.6) Sulfate Chloride -1.03 x 10 (20.6) 1.25 x 10 (20.6) 5.63 x 10 (20.6) 8.34 x 10 (20.6) NA (20.6) NA (20.6) Ion 0.636 0.596 0.242 0.733 <0.001 (20.6)
Sulfate Chloride 0.0167 0.0167 1.23 x 10 ⁻⁷ 2.43 x 10 ⁻⁶ 0.000769 8.25 x 10 ⁻⁷ -0.000172 -0.000154 -0.000769 0.000154 -0.000172 -0.000172 -0.000154 -0.000154 -0.000769 0.000154 -0.000172 -0.000172 -0.000172 -0.000172 -0.000154 -0.000164 -0.000164 0.000154 -0.000172 -0.000172 -0.000172 -0.000154 -0.000154 -0.000164 -0.000154 -0.000164 -0.000164 0.000154 -0.000172 -0.000172 -0.000172 -0.000172 -0.000172 -0.000172 -0.000172 -0.000172 -0.000174 -0.000172 -0.000174 -0.000174 -0.000174 -0.000172 -0.000174 -0
Sulfate -1.55 x 10 ⁻⁶ -1.28 x 10 ⁻¹¹ -3.32 x 10 ⁻⁸ -9.01 x 10 ⁻¹¹ NA c NA c Chloride -1.03 x 10 ⁻⁶ 1.25 x 10 ⁻¹¹ 5.63 x 10 ⁻⁸ 8.34 x 10 ⁻¹¹ NA c NA
B x Ion 0.314 0.041 b 0.018 b 0.067 0.016 b 0.002 b
A x Ion 0.414 0.003 b 0.010 b 0.008 b NA c NA c

Mesocotyl Emergence 70.0 50.0 30.0 20.0 Sulfate ■Chloride treatments

0 1,000 2,000 3,000 4,000 5,000 6,000 7,000 8,000 9,000 Conductivity (umhos/cm) Figure 1. Mesocotyl emergence as a function of conductivity imparted by sulfate- and

chloride-based salts





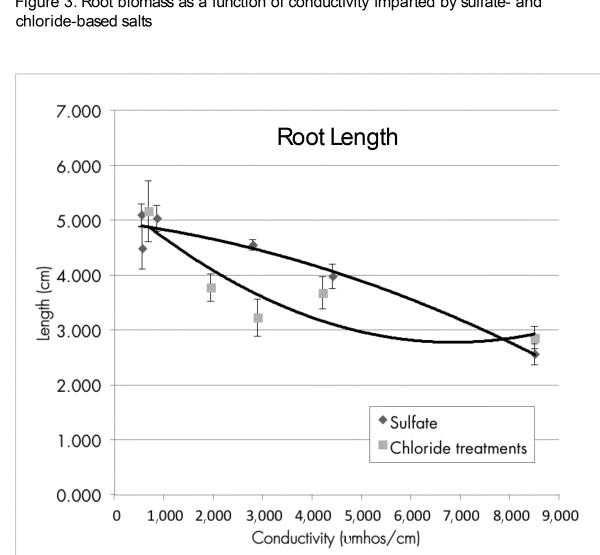
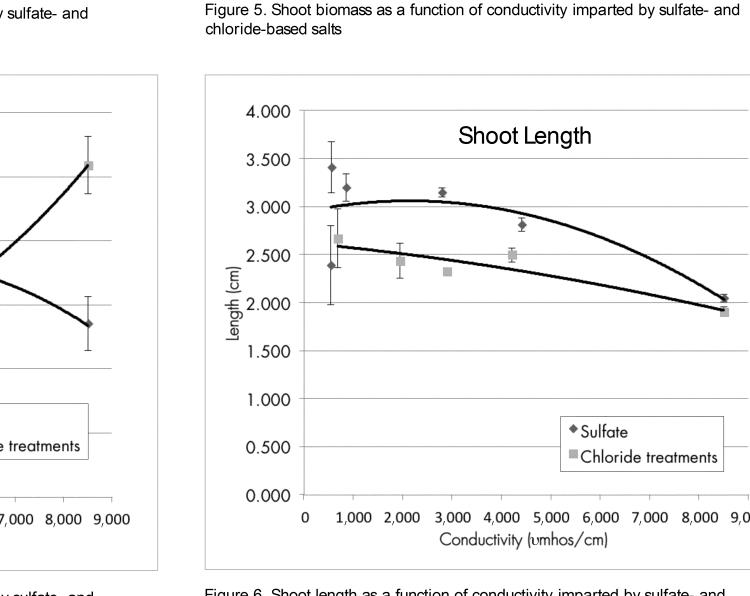


Figure 4. Root length as a function of conductivity imparted by sulfate- and

chloride-based salts

Figure 3. Root biomass as a function of conductivity imparted by sulfate- and



chloride-based salts

0.0140

0.0120

₹ 0.0060

0.0040

0.0020

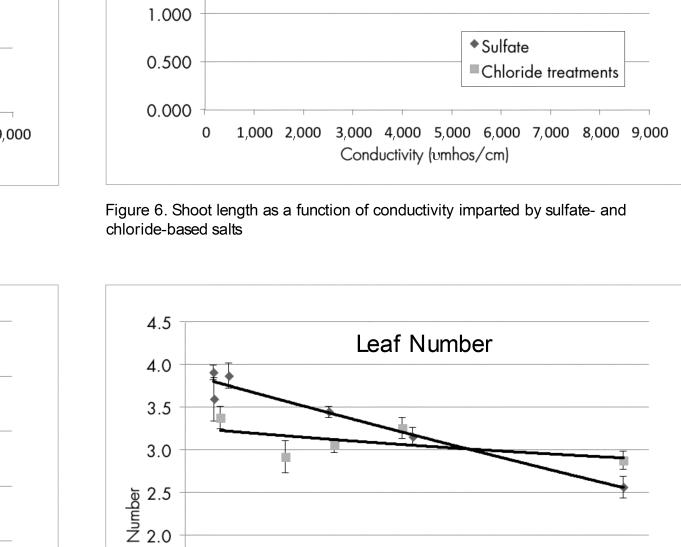
0.0000

Shoot Biomass

0 1,000 2,000 3,000 4,000 5,000 6,000 7,000 8,000 9,000

Conductivity (umhos/cm)

Chloride treatments



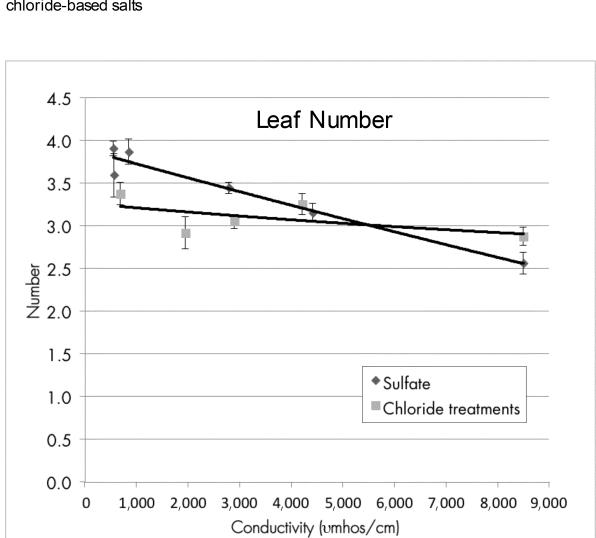


Figure 7. Leaf number as a function of conductivity imparted by sulfate- and

• Root length and phytotoxicity appeared to be especially sensitive to chloride toxicity. • Sulfate was stimulatory to wild rice at some concentrations. Germinated Wild Rice

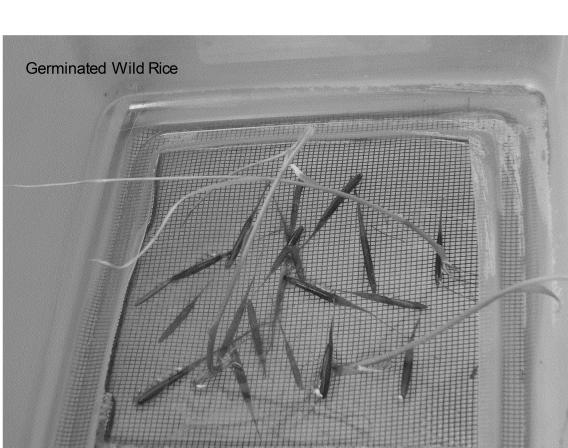
treatments, suggesting effects were due to general osmotic stress and not sulfate toxicity.

Shoot length and leaf number appeared to have sulfate-specific toxic responses.

SUMMARY

CONCLUSIONS

mg /L over the 21-day exposure.



Results from this study indicated that increased sulfate concentrations did not induce an adverse response in wild rice

determined by ET30 assessments, a more rapid germination time occurred at 2,500 mg/L, an apparently anomalous

effect given the lack of stimulation or toxicity in all sulfate exposures. Assessing toxic effects based on IC25 values for

conductivity indicated that sulfate toxicity occurred in the same four test endpoints identified as responding to sulfate

based on LOEC assessments. These endpoints included shoot length, root length, leaf number and phytotoxicity. The

were the most sensitive wild rice endpoints for toxic effects due to sulfate, and that root length was also an especially

sensitive endpoint by which to asses chloride toxicity. Overall results indicate that tissue length endpoints may be more

reduced at 5,000 mg/L sulfate, and multiple regression analysis for both endpoints suggested that osmotic and sulfatespecific effects were likely. In the case of shoot length, the IC25 value was significantly lower for sulfate-treated wild

sensitive measures of growth than biomass endpoints. Neither root biomass nor shoot biomass were significantly

• Sulfate did not adversely affect germination and early development of wild rice at concentrations below 5,000

• Some effects induced at high sulfate concentrations were also observed in osmotically equivalent chloride

influenced by sulfate concentrations of 5,000 mg/L. In contrast, shoot length and root length were significantly

rice than for chloride-treated plants, suggesting that the sulfate-specific effect was predominant.

IC25 determinations also indicated that three endpoints were responsive to chloride toxicity, with root length and phytotoxicity showing greater sensitivity to chloride than to sulfate. Findings suggest that shoot length and root length

seedlings under hydroponic conditions at concentrations below 5,000 mg/L, as determined by LOEC findings. As

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